firefly® application note
Illumina DNA Prep

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Overview

The Illumina DNA Prep kit is an optimized, user-friendly workflow for preparing normalized and ready-to-sequence libraries in under four hours, from a wide range of DNA inputs. Illumina DNA Prep delivers reliable results - consistent insert sizes, uniform coverage, and optimized performance for a range of sample types and DNA input amounts[1,2].

Illumina DNA Prep relies on bead-based tagmentation technology where magnetic bead-based transposome complexes fragment DNA and ligate adapter sequences in a single 15-minute reaction. PCR is then used to add indexes to the DNA fragments and generates libraries compatible with all Illumina sequencing platforms. The amplified libraries are purified using Illumina Purification Beads. The overall process eliminates the need for library quantitation before library pooling and sequencing, minimizing bias and error, and resulting in highly reproducible sequencing data[1,2].

Here we show how the Illumina DNA Prep workflow, based on Illumina DNA Prep Reference Guide (1000000025416 v11), was automated on firefly®, in a two-part protocol with a single off-deck thermocycler step. Details of the firefly® workflow are described, and data is presented to demonstrate the performance of the automated workflow.

Reagents required

Kit	Catalog #
Illumina DNA Prep (96 Samples)	20060059
Illumina DNA/RNA UD Indexes Set A, B, C or D, Tagmentation (96 Indexes, 96 Samples)	20091654 20091656 20091658 20091660

firefly® protocols

Protocol number	Protocol name	Instrument run time
Protocol 1 of 2	Illumina DNA Prep (96) Part 1	1 hour 26 mins
Protocol 2 of 2	Illumina DNA Prep (96) Part 2	1 hour 15 mins

Table 1. firefly® protocols used to run the Illumina DNA Prep workflow – and are available to download from the firefly® community.

firefly® workflow

DNA Input 1-500 ng	Tagment genomic DNA	Post Tag. cleanup	PCR Setup	PCR *Off-deck*	Clean up Libraries
Run time	40 mins	40 mins	6 mins	36-57 mins	75 mins

Figure 1. Workflow and firefly® run times for the Illumina DNA Prep kit.

Workflow features

Minimal user interactions

- Users are only needed to set up the firefly® protocols and to transfer the plate to an off-deck thermocycler to run the PCR program.

The use of plastic consumables has been minimized

- Non-contact positive displacement technology of the firefly® dispense head enables the same reagent to be dispensed to multiple wells of a plate using a single syringe.
- Pipetting-head tips have been reused where appropriate to do so, with no detrimental effect on the library preparation.

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Protocol overview

Protocol 1 of 2
DNA Prep (96) Part 1

Figure 2 shows the starting deck layout for this protocol. The firefly® dispense head is used to dispense a mastermix of Bead-Linked Transposome (BLT) and Tagmentation Buffer 1 (TB1) to the TAG/PCR plate containing the input DNA. Each sample well is tip-mixed then the plate is moved to the pre-heated on-deck thermal module for the Tagmentation incubation (55°C for 15 minutes).

On completion of this incubation, the dispense head delivers Tagmentation Stop Buffer to each sample well of the TAG/PCR plate then the pipetting head tip-mixes each sample well. The TAG/PCR plate is moved back to the thermal module and incubated at 37°C for 15 minutes. After this incubation, the BLT beads are washed three times with Tagmentation Wash Buffer (TWB). The TWB is dispensed by syringes 1, 2 and 3 of the firefly® dispense head then is tip-mixed. The same set of tips is used for each bead resuspension and supernatant removal of the TWB washes.

Diluted Enhanced PCR Mix (EPM) is dispensed to the TAG/PCR Plate using the dispense head, then Indexes are transferred to the sample plate using the pipetting head, and each sample well is tip-mixed. The user moves the TAG/PCR plate from deck position U4 and to a thermocycler pre-programmed with the BLT PCR program (Table 2). On completion of the PCR program the sample plate is ready to proceed to the Illumina DNA Prep (96) Part 2 protocol or be stored at 2°C to 8°C for up to 30 days.

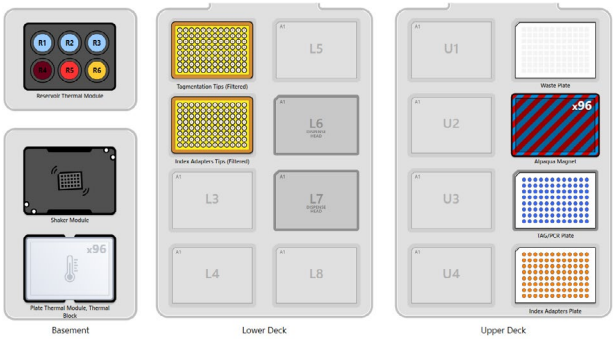

Figure 2. Starting deck layout for firefly® Illumina DNA Prep (96) Part 1. Lower deck: (L1) Tagmentation Tips (Filtered) - 100µL pipetting head tips; (L2) Index Adapters Tips (Filtered) - 100µL pipetting head tips. Upper deck: (U5) Waste Plate - Abgene 1.2mL Deep Well Thermo Fisher Scientific; (U6) Alpaqua 96S Super Magnet; (U7) TAG/PCR Plate - Hard Shell Plate Bio-Rad with 30µL input DNA per well; (U8) Index Adapters Plate - twin.tec PCR Eppendorf plate. Dispense head reservoirs: (R1-3) Tagmentation Wash Buffer; (R4) Tagmentation Buffer 1 and Bead-linked Transposomes 1:1 Master Mix; (R5) Tagmentation Stop Buffer; (R6) Enhanced PCR Mix and Nuclease-free Water 1:1 Master Mix. Plate Thermal Module: 96 Thermal Block.

Step	Temperature (°C)	Time (seconds)	Cycles
1	68	180	
2	98	180	
3	98	45	12 for 1-9ng input 8 for 10-24ng input 6 for 25-49ng input 5 for 50-500g input
4	62	30	
5	68	120	
7	68	60	
8	10	Hold	

Table 2. BLT PCR program[2].

Protocol 2 of 2
DNA Prep (96) Part 2

On completion of the BLT PCR thermocycler program, the user returns the TAG/PCR Plate to position U4 on the firefly® deck and starts this Illumina DNA Prep (96) Part 2 protocol. This protocol performs a double-sided bead purification of the tagmented, indexed, amplified libraries. See Figure 3 for the starting deck layout.

Whilst the TAG/PCR Plate is incubated on a magnet, the firefly® dispense head is used to dispense Illumina Purification Beads (IPB) to IPB Plate 1 and IPB Plate 2, then dispenses Nuclease-free water to IPB Plate 1. PCR supernatant from the TAG/PCR Plate is transferred to IPB Plate 1, and is tip mixed with IPB which was dispensed to the wells earlier. After a room temperature incubation followed by an on-magnet incubation, the IPB 1 supernatant is transferred to IPB plate 2 and is tip-mixed with the IPB and water which was dispensed to this plate earlier. After a room temperature incubation followed by an on-magnet incubation, the IPB 2 supernatant is removed to the Waste Plate.

Beads in IPB Plate 2 are washed twice with 80% Ethanol dispensed from three syringes of the dispense head. The pipetting head is used for all Ethanol removal steps and the same set of tips is reused for these washes.

After air-drying the beads for 5 minutes, the dispense head is used to dispense Resuspension Buffer to IPB Plate 2. The pipetting head is used to tip-mix samples to resuspended the beads. IPB plate 2 is incubated at room temperature then on a magnet before the supernatant, containing the eluted library DNA, is transferred, into the Final Libraries Plate.

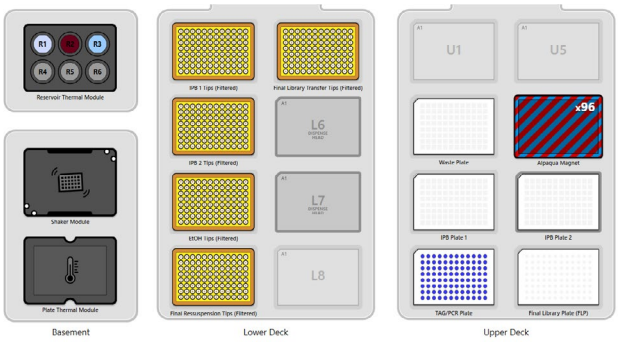

Figure 3. Starting deck layout for firefly® Illumina DNA Prep (96) Part 2. Lower deck: (L1) IPB 1 Tips (Filtered) - 100µL pipetting head tips; (L2) IPB 2 Tips (Filtered) - 100µL pipetting head tips, (L3) EtOH Tips (Filtered) - 100µL pipetting head tips; (L4) Final Resuspension Tips (Filtered) - 100µL pipetting head tips; (L5) Final Library Transfer Tips (Filtered) - 100µL pipetting head tips. Upper deck: (U2) Waste Plate - Abgene 1.2mL Deep Well Thermo Fisher Scientific; (U3) IPB Plate 1 - Abgene 1.2mL Deep Well Thermo Fisher Scientific; (U4) TAG/PCR Plate - Hard Shell Plate Bio-Rad with 50µL BLT PCR reaction mix per well; (U6) Alpaqua 96S Super Magnet; (U7) IPB Plate 2 - Abgene 1.2mL Deep Well Thermo Fisher Scientific; (U8) Final Library Plate - Hard Shell Plate Bio-Rad. Dispense head reservoirs: (R1) Nuclease-free Water; (R2) Illumina Purification Beads; (R3) Resuspension Buffer; (R4-6) 80% Ethanol.

Protocol performance

Input titration and high throughput performance

The Illumina DNA Prep kit was run on firefly® using human genomic DNA (Roche, part 11691112001) at 1ng, 35ng, 100ng, and 500ng inputs, with 14 replicates and two no-template controls (NTC) per input. The BLT PCR program was run on an external thermocycler with 12 and 6 cycles for the 1ng and 35ng inputs respectively. For the 100ng and 500ng inputs, the PCR program was run using 5 cycles.

A full plate of 96 samples was processed using the Illumina DNA Prep kit and human genomic DNA NA12878 (Coriell Institute for Medical Research) at 100ng input per sample and 5 cycles of PCR (see Table 3), from which 16 final libraries were randomly selected and sequenced on a NovaSeq™ 6000 Sequencing System, with a run configuration of 2 × 151 bp and an S4 flow cell.

Run type	gDNA Input (ng)	Source	Sample size (n)	PCR cycles
Input titration	1	Roche 11691112001	14	12
	35	Roche 11691112001	14	6
	100	Roche 11691112001	14	5
	500	Roche 11691112001	14	5
High throughput	100	Coriell NA12878	96	5

Table 3. Run set-up used to assess the performance of the Illumina DNA Prep workflow on firefly®.

Library concentration was determined using the KAPA Library Quantification kit and a Lightcycler® 480 (Roche). The fragment size distribution for each library was determined using the HS NGS Fragment kit and a Fragment Analyser (Agilent).

Figure 4 shows that libraries produced from gDNA inputs ranging from 35ng to 500ng have a concentration distributed between 20nM and 40nM, with the libraries generated from the 1ng input achieving a final concentration of 70nM. Figure 5 demonstrates that the libraries prepared using the firefly® automated Illumina DNA Prep workflow have a reproducible fragment size distribution between 500 and 600bp (%CV < 5%). The high throughput run performed using of the firefly® protocol demonstrates that library concentration and average fragment sizes are uniform across a 96-well plate (%CV < 10%), see Table 4 and Table 5.

The library yield, for inputs >35ng, and library fragment size distribution are comparable to those of libraries produced manually with the Illumina DNA Prep kit[3]. Table 6 summarizes the sequencing performance of a pool of 16 libraries generated from 100ng Coriell NA12878 gDNA on firefly®. All metrics meet their respective criteria.

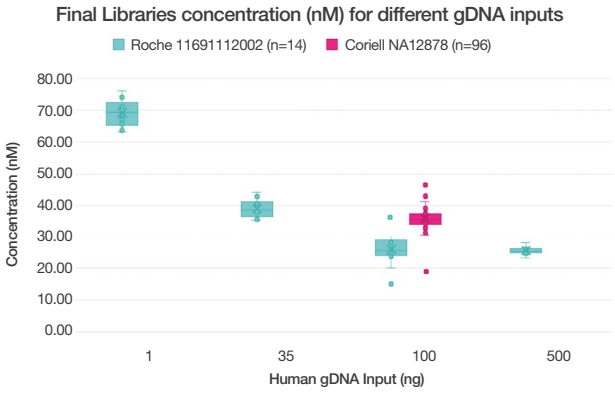

Figure 4. Final libraries concentration output as a function of genomic DNA input and source. All samples were processed on firefly® using the Illumina DNA Prep kit. Sample size: Roche Human gDNA (blue bars), 14 replicates; Coriell NA12878 (orange bar), 96 replicates.

Figure 5. Final libraries fragment size output as a function of genomic DNA input and source. All samples were processed equally on firefly® instrument using the Illumina DNA Prep kit. Sample size: Roche Human gDNA (blue bars), 14 replicates; Coriell NA12878 (orange bar), 96 replicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	33.6	34.7	35.3	34.2	33.6	37.6	36.2	38.5	37.7	32.4	33.9	36.2
B	43.0	38.5	38.4	35.8	35.3	39.1	37.4	36.6	34.0	34.7	36.0	37.2
C	37.5	34.1	33.6	33.4	35.5	35.5	36.2	34.0	36.6	32.6	36.0	36.6
D	18.8	33.9	31.4	33.9	33.7	36.8	35.4	33.5	33.9	31.6	37.8	38.0
E	36.6	31.5	34.9	33.2	34.1	37.5	37.4	35.6	36.1	36.6	34.4	34.5
F	34.4	35.6	38.2	35.4	34.9	46.7	35.5	37.3	34.5	35.8	44.1	38.1
G	33.5	35.3	36.9	33.0	35.6	41.2	37.2	39.1	36.0	33.9	33.6	34.2
H	36.1	30.7	32.8	38.5	46.5	47.8	39.8	43.1	38.4	34.9	33.1	37.9

Table 4. Final library concentration (nM) across a 96-well plate. Libraries generated from 100ng input of Coriell NA12878. %CV = 9.96%.

	1	2	3	4	5	6	7	8	9	10	11	12
A	554	543	541	536	545	541	546	526	543	548	538	546
B	541	546	547	544	539	541	541	540	542	540	549	533
C	538	547	540	544	545	541	539	543	543	543	542	555
D	545	538	554	546	543	543	539	542	549	538	544	535
E	537	546	548	543	549	542	537	545	547	548	541	540
F	546	542	548	538	548	548	547	535	545	545	546	549
G	540	542	542	556	536	543	545	541	547	549	542	553
H	533	535	536	537	534	533	532	525	541	541	549	544

Table 5. Average library fragment size (bp) across a 96-well plate. Libraries generated from 100ng input of Coriell NA12878. %CV = 1.04%.

Metrics	Criteria	Result
S4 Flow Cell		
Yield	>2400Gbp	3539Gbp
%PF	≥70%	72.66%
%Q30	≥85%	88.34%
Index %CV	≤20% with correction factors	14.29%
Samples (Average)		
Yield	≥100Gbp for ≥95% of samples	195.6Gbp
Autosomal coverage	≥30x for ≥95% of samples	56.89x
Insert size	300-350bp	317.8bp
Q30 bases (excluding duplicates & clipped bases)	≥85Gbp for ≥95% of samples	158.5Gbp
Duplicate read rates	≤15% for ≥95% of samples	8.99%
Percentage of genome with at least 15x coverage	≥90% for ≥95% of samples	93.72%

Table 6. Sequencing metrics for 16 libraries run on a NovaSeq™ 6000 with an S4 flow cell. All libraries were prepared on firefly® using 100ng of Coriell NA12878 DNA.

Cross Contamination Evaluation

The occurrence of well-to-well contamination was evaluated by preparing a total of 96 libraries on firefly®: 48 replicates of a 100ng gDNA input (Roche, 11691112001) and 48 no-template controls (NTC) interspersed in a checkerboard pattern (Table 7). The samples and NTC were processed using the Illumina DNA Prep kit and 5 cycles of PCR.

Well-to-well variability was assessed by measuring the fluorescence of all 96 samples using a fluorescent DNA-binding dye (Promega, QuantiFluor® dsDNA) and a FLUOstar Omega plate reader (BMG Labtech). The fragment size distribution was also measured for each well of the plate, using a Fragment Analyser and HS NGS Fragment kit (Agilent).

[1] *For life science research only. Not for use in diagnostic procedures.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	-	+	-	+	-	+	-	+	-	+	-
B	-	+	-	+	-	+	-	+	-	+	-	+
C	+	-	+	-	+	-	+	-	+	-	+	-
D	-	+	-	+	-	+	-	+	-	+	-	+
E	+	-	+	-	+	-	+	-	+	-	+	-
F	-	+	-	+	-	+	-	+	-	+	-	+
G	+	-	+	-	+	-	+	-	+	-	+	-
H	-	+	-	+	-	+	-	+	-	+	-	+

Table 7. Checkerboard pattern with 100ng DNA input (+) and NTC (-) wells.

Table 8 shows that high fluorescence readings (>15000 RFU) were obtained in all 48 wells containing gDNA input with high reproducibility (%CV = 5.4%), while all 48 NTC wells showed negligible fluorescence (<20 RFU). The fragment analyser traces in Figure 6B show the positive gDNA sample wells to have an average fragment size of 556bp and a %CV of 1.2%, which are in line with reported traces for libraries produced manually with the Illumina DNA Prep kit[3]. Fragment analyser traces for all 48 NTC wells (Figure 6A) show an absence of any library peak, indicating that the no well-to-well contamination was detectable in the firefly® run.

	1	2	3	4	5	6	7	8	9	10	11	12
A	17914	<20	17175	<20	15911	<20	19313	<20	17443	<20	17475	<20
B	<20	16642	<20	17006	<20	16841	20	16894	<20	16405	<20	18432
C	17263	<20	17319	<20	17268	<20	17793	<20	16051	<20	15543	<20
D	<20	17305	<20	18159	<20	18909	<20	18021	<20	17918	<20	17688
E	15112	<20	17307	<20	17200	<20	17137	<20	17711	<20	16403	<20
F	<20	18241	<20	17039	<20	19272	<20	18254	<20	17681	<20	16379
G	15981	<20	16566	<20	17945	<20	17810	<20	18456	<20	17383	<20
H	<20	18322	<20	18229	<20	18938	<20	18850	<20	17694	<20	16523

Table 8. Average fluorescence values (RFU) of 48 gDNA positive wells (magenta) interspersed with 48 NTC controls (grey). All wells were measured in triplicate and the blank-subtracted.

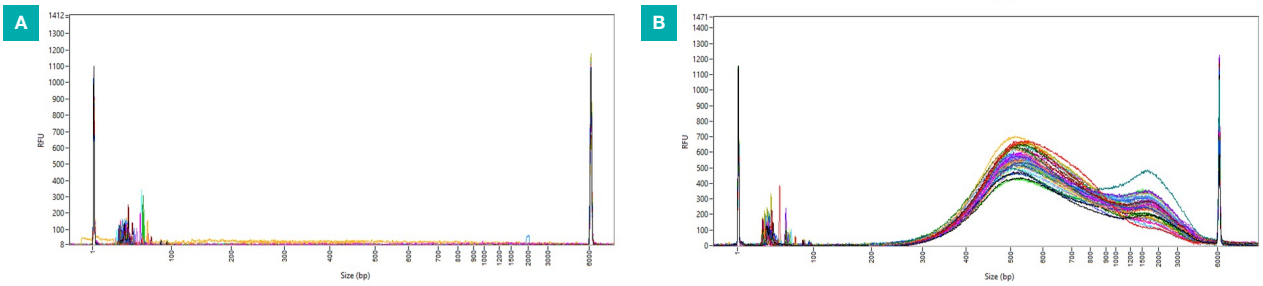

Figure 6. Fragment Analyser traces of 48 NTC controls (A) interspersed with 48 gDNA positive wells (B).

Summary

The data presented here demonstrates that high quality libraries, with consistent library concentration and fragment size, can be prepared on firefly® using the Illumina DNA Prep kit.

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References

(1) Illumina DNA Prep Data Sheet (M-GL-01373 v1.0)

(2) Illumina DNA Prep Reference Guide (1000000025416 v11), published 17 February 2025.

(3) Bruinsma, S., Burgess, J., Schlingman, D. et al. Bead-linked transposomes enable a normalization-free workflow for NGS library preparation. BMC Genomics 19, 722 (2018). https://doi.org/10.1186/s12864-018-5096-9.